Mechanisms of antimicrobial actions of quaternary ammonium compounds

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Effect of different cationic antimicrobials on the cell surface structure of adhering staphylococci

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Abstract

Initial effects of different cationic antimicrobials ((Cocoalkyl methyl (polyoxyethylene) ammonium chloride (QAC), Gramicidin S and gentamicin sulphate) on the cell surface structure of adhering *Staphylococcus epidermidis* were investigated and compared using Peak-Force-Tapping Atomic Force Microscopy (AFM). Adhering *S. epidermidis* ATCC 14990 was exposed to different antimicrobials at their minimal bactericidal concentration and to potassium phosphate buffer, as a control. During exposure, adhering bacteria were repeatedly imaged applying a force of 3 nN and the percentage of staphylococci that remained adhering was registered as a function of time. Surface roughness of bacteria that remained adhering was calculated from the AFM images. Upon progressive exposure of adhering staphylococci to all three cationic antimicrobials, wrinkling and disappearance of bacteria was observed during scanning with the AFM tip. Exposure to buffer did not yield wrinkling or disappearance of adhering staphylococci, while after 300 min more bacteria had detached when exposed to gentamicin sulphate than to the other two cationic antimicrobials. Bacterial cell surface roughness after exposure to the antimicrobials and scanning increased from 15 nm prior to the experiment to 138 nm for QAC and Gramicidin S and to 145 nm for gentamicin sulphate. The bacterial cell surface roughness was affected upon exposure to the antimicrobial compounds, which confirms that membrane degradation is a significant contributing factor to their bactericidal activities. Antimicrobial effects on the cell surfaces of adhering staphylococci were found to be similar for a quaternary ammonium compound, an antimicrobial peptide and aminoglycoside, suggesting similar membrane interactions.

Introduction

The spread of infections in healthcare settings is often difficult to control due to the presence of multiple intrinsic and acquired mechanisms of antimicrobial resistance [2-4]. A wide range of antibiotics, disinfectants and detergents is currently being explored to control bacterial adhesion, biofilm formation, and subsequent infection. Bacteria in a biofilm that cause infections can develop various ways to survive drugs meant to kill or weaken them and frighteningly, bacterial ingenuity to survive antibiotic treatment is increasing with the extended use or misuse of antibiotics. Despite considerable variation in composition, structure, and putative mechanism of action, many antimicrobials initially interact with bacterial cell wall membranes to become integrated in the membrane. The interaction of an antimicrobial with the cell membrane can be governed by hydrophobic or electrostatic interactions [1]. Since the bacterial cell surface is usually negatively charged under physiological conditions [10], cationic antimicrobials all have favourable electrostatic interactions with bacterial cell surfaces. Among the known cationic antimicrobials are frequently used antibiotics, such as gentamicin sulphate and antimicrobial peptides, such as Gramicidin S.

In this respect, positively charged Quaternary Ammonium Compounds (QACs) may be regarded as the synthetic analogues of antimicrobial peptides. QACs have a comparable molecular mass as several antibiotics and antimicrobial peptides and are effective on both Gram-positive and Gram-negative bacteria [4,15,20]. QACs interact with bacterial cell surfaces to perturb the bacterial cell membrane and affect its integrity by creating holes, followed by leakage of intracellular constituents, ultimately leading to cell death [3,17,18,25]. Gramicidin S is a naturally occurring cationic, antimicrobial peptide, believed to play a major role in innate host defence against infection. Its mechanism of action is probably similar as of QACs. Gentamicin sulphate is one of the most

commonly used broad-spectrum antibiotics in the anti-infective armamentarium [11], also disrupting bacterial cell membranes and killing bacteria by inhibiting protein synthesis through binding to the 16S rRNA [22].

Recently [2], we demonstrated that repeated imaging of adhering staphylococci with atomic force microscopy (AFM) in the contact mode during exposure to a QAC caused wrinkling of the cell surface until bacteria disappeared from the substratum during scanning. Staphylococci adhering in un-scanned areas remained adhering during exposure to QAC, which suggests that stress deactivation [14] caused by the pressure of the AFM tip assisted incorporation of QAC molecules in the membrane and enhanced their bactericidal efficacy. Although acquisition of AFM images in the contact mode is commonly used, providing high-resolution images, the continuous direct contact between the tip and sample causes significant lateral forces, which can distort soft biological samples.

Therefore, it is the aim of this study to apply Peak Force Tapping (Quantitative NanoMechanics) AFM to compare the initial effects of different cationic antimicrobials, which display distinctly different structures. (Cocoalkyl methyl (polyoxyethylene) ammonium chloride, Gramicidin S and gentamicin sulphate) on the cell surface structure of a *Staphylococcus epidermidis* strain. Peak Force Tapping precisely controls the imaging force, keeping indentations small (less than several nanometers in most cases) to prevent sample damage and maintains high-resolution imaging at the same time.

Materials and methods

Bacteria and culture conditions

S. epidermidis ATCC 14990, originating from a nose infection, was used in this study. The strain was first streaked on a blood agar plate from a frozen stock solution (7% (v/v) DMSO) and grown overnight at 37°C on a blood agar plate. One colony was inoculated in 10 ml tryptone soy broth (TSB, Oxoid, Basingstoke, UK) and incubated for 24 h at 37°C in ambient air. This culture was used to inoculate a second culture of 200 ml, which was grown for 16 h under the same conditions. Bacteria were harvested by centrifugation at 6500 g for 5 min at 10°C, washed twice with demineralised water and suspended in 10 ml of demineralised water for AFM analysis.

Antimicrobials

Ethoquad C/25, Cocoalkyl methyl (polyoxyethylene) ammonium chloride (molecular mass 910 g/mol) was obtained from AKZONobel (Amsterdam, The Netherlands) and used as received (Figure 1A). Gramicidin S (Figure 1B; molecular mass 1141 g/mol) has been kindly provided by Bio-Organic Synthesis, Leiden Institute of Chemistry, University of Leiden, The Netherlands and was used as received. Gentamicin sulphate (Sigma-Aldrich) (molecular mass 478 g/mol; Figure 1C) was diluted with sterile demineralised-water to obtain fourfold stock solutions of 32 µg/ml, respectively.

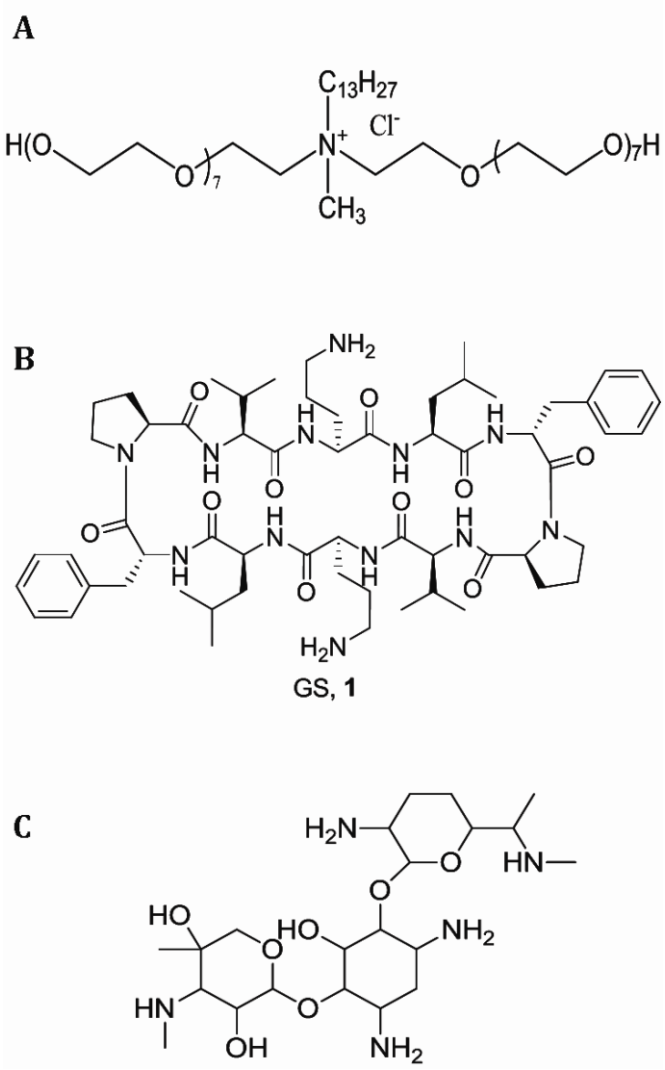

Figure 1. Chemical structure of: (A) QAC (Cocoalkyl methyl (polyoxyethylene) ammonium chloride), (B) Gramicidin S, (C) gentamicin sulphate.

Minimal inhibitory (MIC) and bactericidal (MBC) concentrations

The antimicrobial activities (MIC and MBC) of the cationic antimicrobials were determined against *S. epidermidis* ATCC 14990. Briefly, the wells of a sterile 96 tissue culture plate (Falcon, USA) were filled with 100

µl of different concentrations of the antimicrobials in TSB to which 100 µl of a diluted bacterial pre-culture was added. A potassium phosphate buffer (10 mM, pH 7.0) was used as a control. The plates were incubated for 24 h at 37°C. The lowest concentration of the antimicrobial agent that completely inhibited visual bacterial growth was defined as MIC. The MBC was obtained by adding a droplet of 10 µl from each well from the microtiter plate showing no visible growth, on a TSB agar plate. Subsequently, the concentration showing no visible growth was taken as the MBC. MIC and MBC were determined three times with separately cultured bacteria.

Table 1. MIC and MBC values (mg/ml) of QAC, Gramicidin S and gentamicin sulphate in 10 mM potassium phosphate buffer (pH 7) for planktonic *S. epidermidis* ATCC 14990 in TSB.

Compound	MIC	MBC
QAC	0.055	0.110
Gramicidin S	0.001	0.002
Gentamicin sulphate	0.004	0.008

Atomic force microscopy

AFM imaging was done at room temperature in 10 mM potassium phosphate buffer (pH 7.0) as a control and with QAC, Gramicidin S and gentamicin added in different concentrations. For bacterial imaging, a Nanoscope V AFM (Brüker) was used, operating in the Peak Force Tapping (Quantitative NanoMechanics) mode. "V"-shaped silicon nitride cantilevers from Brüker with a spring constant of about 0.5 N/m and a probe curvature

radius of 20 nm were calibrated for each experiment, according to the manufacturer's specifications.

Staphylococci were immobilized on positively-charged glass slides, after thorough cleaning and adsorption of poly-L-lysine (0.1% solution, SIGMA, United Kingdom). In brief, 1 ml of a bacterial suspension (10^{10} bacteria/ml) was allowed to adhere for 30-40 min on the coated glass slides and finally rinsed with demineralised water to remove free floating bacteria and leaving immobilized staphylococci.

Peak Force Tapping mode topographic images of bacteria immobilized on the glass slides were taken with a scan size of 35 μm x 35 μm at the beginning and end of an experiment, usually lasting 10-15 min. In the centre of these images, an 8 μm x 8 μm area was continuously scanned during 300 min. All scans were made with an applied force of 3 nN, at a scan rate of 0.3 Hz.

The roughness (R_a) of individual staphylococci prior and after exposure to the cationic antimicrobials was determined from height images of the adhering bacteria. Note that this analysis could only be carried out for bacteria that maintained adhering to the surface after repeated scanning. Images were flattened and plane fitted prior to analysis and roughness calculated for a rectangular region of 345 x 345 nm on the top of each bacterium.

Fluorescence microscopy

In a separate experiment, cell surface-damaged of immobilized bacteria on the glass slides immediately after preparation as described above and after 60 min exposure to the antimicrobials solution or potassium phosphate buffer (control), were investigated in the absence of AFM scanning, using fluorescence microscopy. The samples were staining with LIVE/DEAD stain (*Bac*Light Bacterial Viability Kit, 3.34 mM SYTO 9 dye and 20 mM

Propidium iodide). After 15 min in the dark, the samples were analyzed with a fluorescent microscope (Leica DM4000B Microsystems Ltd, Germany). Bacteria with damaged cell membranes were red-fluorescent, whereas bacteria with an intact cell membrane appeared green-fluorescent.

Statistical analyses

Survival of adhering bacteria exposed to QAC, Gramicidin S, gentamicin sulphate and buffer control were compared using the Log-rank (Mantel-Cox) algorithm of the Windows package SPSS 12.0.1. Roughness differences of individual staphylococci prior to and after exposure to the cationic antimicrobials were compared according to a nonparametric Mann-Whitney test, using Graph Pad Prism 5.01 software. Statistical significance was accepted at a confidence level of 95% and $p < 0.05$.

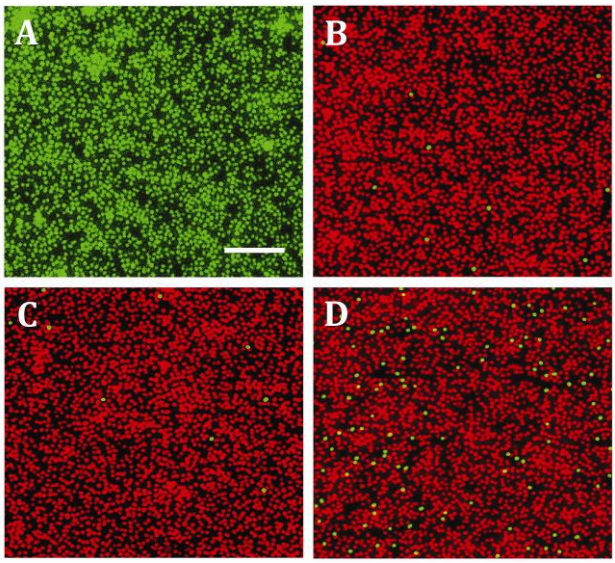

Figure 2. Fluorescence images of *S. epidermidis* ATCC 14990 stained with *Bac*Light LIVE/DEAD stain in a biofilm mode of growth after 60 min exposure to different cationic antimicrobials at their MBC. (A) 10 mM potassium phosphate buffer (control), (B) QAC, (C) Gramicidin S, (D) gentamicin sulphate. The bar denotes 25 µm.

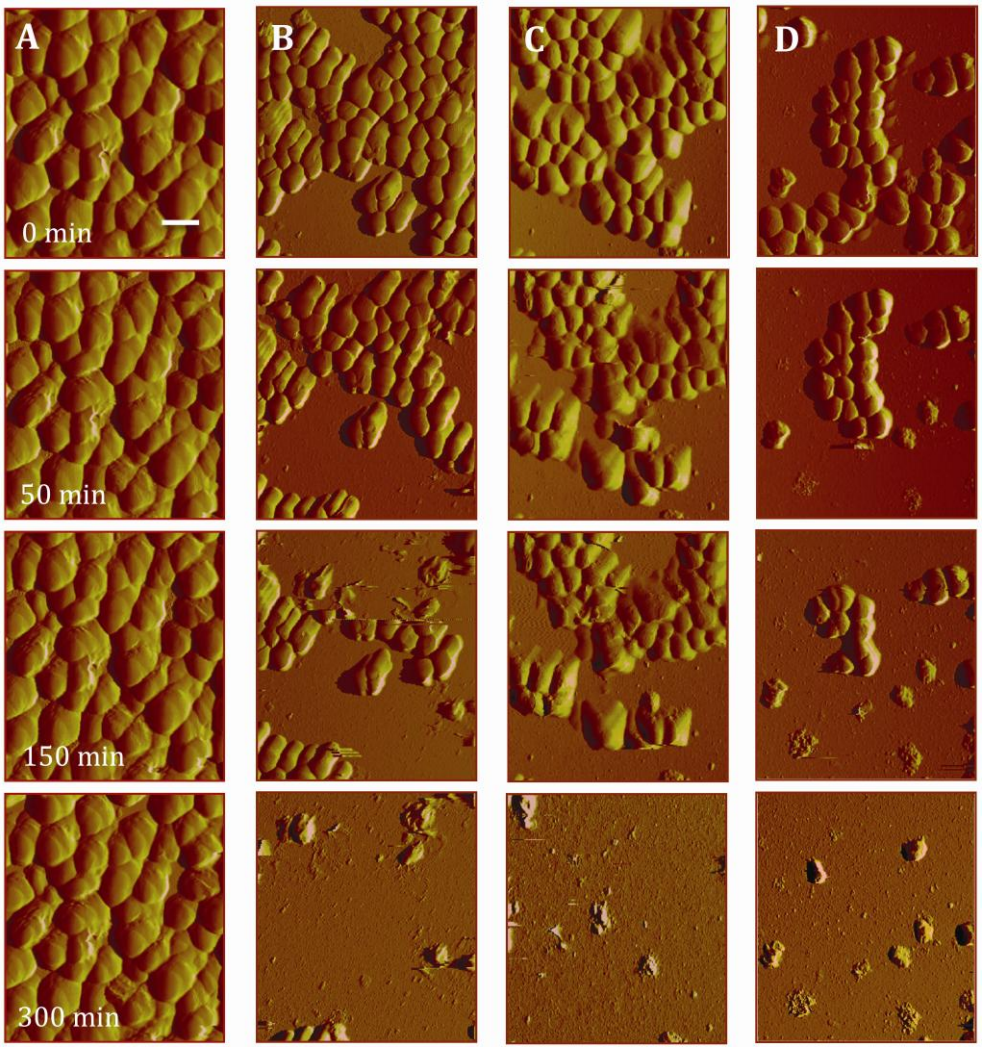

Figure 3. AFM Peak Force Error images of *S. epidermidis* ATCC 14990 during continuous scanning at a rate of 0.3 Hz under an applied force of 3 nN and simultaneous exposure to different cationic antimicrobials at their MBC. (A) 10 mM potassium phosphate buffer (control), (B) QAC, (C) Gramicidin S, (D) gentamicin sulphate. The scan area equals 10 µm x 10 µm. The bar denotes 1 µm.

Results

All cationic antimicrobials included in this study were effective against *S. epidermidis* ATCC 14990 in its planktonic state, as can be seen from the MIC and MBC values collected in Table 1.

In Figure 2, fluorescence images are presented of *S. epidermidis* ATCC 14990 adhering to a glass slide as prepared for AFM measurements and exposed to the different antimicrobials. Exposure to buffer did not negatively impact the viability of the staphylococci, or strictly speaking ''membrane integrity'', but addition of the QAC or Gramicidin S at their MBC caused severe membrane damage within 99% of all bacteria, whereas exposure to 1x MBC gentamicin sulphate yielded membrane damage in 94% of the adhering staphylococci.

Adhering staphylococci progressively disappeared from areas repeatedly scanned with the AFM tip when exposed to the cationic antimicrobials, but not when exposed to buffer (Figure 3). The percentage maintenance of adhering bacteria on the surface is plotted in a Kaplan Meier survival curve (Figure 4) for the different antimicrobials. Both QAC and Gramicidin S caused significant detachment ($p < 0.001$) at their MBC as compared with the control and yielded 65% bacterial removal after 300 min. Gentamicin at its MBC caused the largest detachment after 300 min with a bacterial removal of 97%.

Staphylococcal removal was preceded by severe wrinkling of the cell surface, as can be seen in Figure 5, summarizing the roughness of the staphylococcal cell surface prior to exposure to the antimicrobials and of the staphylococci that managed to remain adhering after exposure and scanning. Note that whereas there was no roughening of the cell surface when exposed to buffer, all three cationic antimicrobials yielded similar wrinkling of the cell surface from a roughness value of 15 nm for the control to around 145 nm for

gentamicin sulphate, and 138 nm for QACs and Gramicidin S, respectively. Bacteria on the side of the scanned area but exposed to buffer or one of the antimicrobials increased slightly to a surface roughness of 20 nm for the buffer control and to around 34 nm, 49 nm and 56 nm for gentamicin sulphate, QAC and Gramicidin S, respectively.

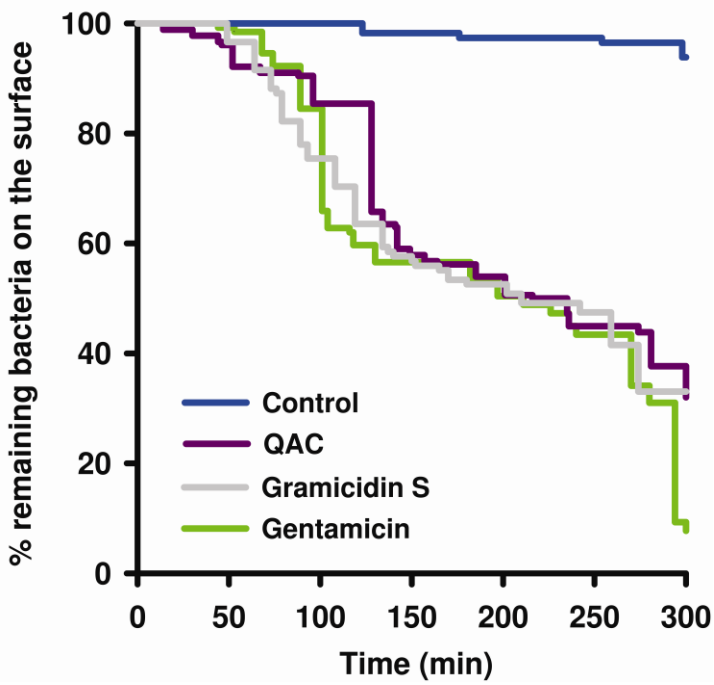

Figure 4. Kaplan Meier curves expressing the percentage maintenance of adhering staphylococci on glass during exposure to QAC, Gramicidin S and gentamicin sulphate in a 10 mM potassium phosphate buffer (pH 7), while scanning continuously at a rate of 0.3 Hz and a 3 nN applied force. Control data refer to potassium phosphate buffer in absence of any cationic antimicrobials.

Discussion

QAC, Gramicidin S and gentamicin exhibited antibacterial activity toward planktonic staphylococci, while also adhering staphylococci were membrane damaged upon exposure to these cationic antimicrobials. In addition, during AFM scanning progressive exposure of adhering staphylococci to QAC, Gramicidin S and gentamicin yielded wrinkling and disappearance of the adhering bacteria from the surface. Staphylococci that remained adhering after AFM scanning had a roughened cell surface, as the onset of wrinkling and final disappearance. Cell surfaces became roughened to approximately the same surface roughness, although more staphylococci detached from the surface during exposure to gentamicin than to the other two cationic antimicrobials. This suggests that these three different cationic antimicrobials initially act in a similar fashion on the bacterial cell surface. In this respect, QACs may thus be regarded as synthetic antimicrobial peptides.

Although they have different applications and structures, QACs, Gramicidin S and gentamicin have been hypothesized to be ”membrane active” agents. Water-soluble low molecular mass QACs are commonly used biocides applied in contact lens care solutions to enhance antimicrobial properties [12] or mouth rinses [23], while Gramicidin S has historically been employed as a topical antibiotic for the treatment of infections of superficial wounds. Gentamicin sulphate, being a heat-stable antibiotic is mainly used for local delivery in the prophylaxis and treatment of orthopaedic implant infections by means of spacers or beads [7,21]. These cationic antimicrobials have in common that they are embedded into the hydrophobic regions of the anionic lipid membranes, thereby causing membrane damage and disintegration [9,19] as demonstrated unequivocally in this manuscript.

Antimicrobial peptides, such as Gramicidin S, possess a tertiary structure in which the cationic groups are spatially located on one side of the

peptide and the hydrophobic moieties at the other side of the molecules [8,16]. Similarly, cationic compounds as QACs display only antibacterial activity if they possess a hydrophobic unit, besides the quaternary ammonium moiety [13]. The resemblance of these requirements for both classes of compounds, to display antibacterial behaviour, suggests a similar way of action.

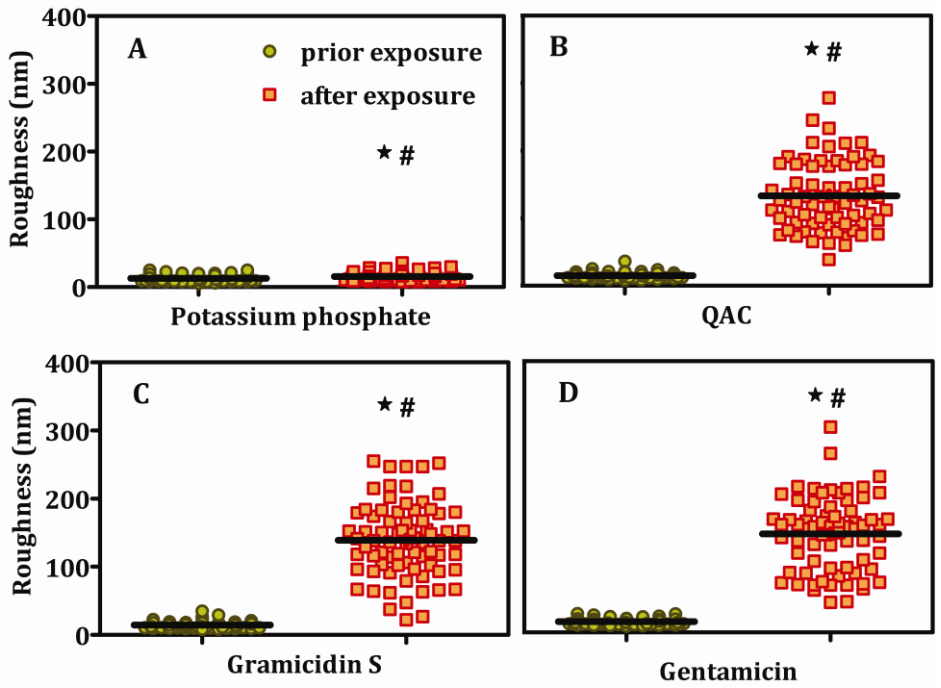

Figure 5. Vertical scatter bars of the cell surface roughness R_a (± SD) of *S. epidermidis* ATCC 14990 immediately upon initial contact and after exposure to different cationic antimicrobials. (A) 10 mM potassium phosphate buffer (control), (B) QAC, (C) Gramicidin S, (D) Gentamicin sulphate. R_a values were measured immediately upon initial contact and after exposure. Each data point corresponds to a single R_a value recorded for one bacterium. Mean roughness values are indicated with a line. Statistically significant differences ($p < 0.05$; Mann-Whitney test) with respect to the bacteria during scanning continuously exposed to potassium phosphate buffer (*) and immediately upon initial contact (#) *versus* bacteria exposed at the beginning of treatment with QAC, Gramicidin S and gentamicin sulphate.

As bacterial membranes are typically negatively charged [10], it is believed that attractive electrostatic interactions play a prime role in the association of positively charged compounds with negatively charged bacterial cell walls. Cationic antimicrobials interact with bacterial cell surfaces to become integrated in the bacterial cell membrane causing the cytoplasmic membrane instability by creating holes, followed by leakage of intercellular material leading finally to cell death. Gram-positive bacteria have a relatively thicker but more porous cell wall made up of inter-connected peptidoglycan layers surrounding a phospholipid cytoplasmic membrane [5]. Phospholipids comprise two long fatty acids connected via glycerol to phosphoric acid with a negative rest charge that is neutralized by divalent cations, such as calcium or magnesium. The displacement of these divalent cations that link the polysaccharides of adjacent lipopolysaccharide molecules by cationic molecules destabilizes the membrane, which results in membrane damage (see fluorescent micrographs in Figure 2) followed by leakage of the intracellular matrix of a bacterium and loss of turgor pressure. Loss of turgor pressure is evidenced by the wrinkling of the staphylococcal cell surfaces and a decrease in bacterial volume (see AFM images in Figure 3), similar as observed after bacterial exposure to antimicrobial peptides [6].

In a previous study [2], we suggested that the incorporation of QACs in the membrane, leakage associated loss of turgor pressure and subsequent removal from the surface were accelerated by external stress, as arising from the substratum and enhanced by the force exerted by the AFM tip. As a major difference with our previous study, we here apply peak force tapping AFM that not only precisely controls the imaging force, but also keeps indentations by the AFM tip to a minimum, reducing stress deactivation by the AFM tip. Still the damage can be clearly seen (Figure 3). Thus, it can be concluded that stress deactivation speeds up the incorporation of cationic antimicrobials, which is confirmed by a 3-4 times higher cell surface roughness of the bacteria

under continuously scanning compared to the roughness of bacteria exposed to the antimicrobials outside the scan area. Since the application of peak force tapping AFM reduces sample damage and maintains high-resolution imaging at the same time, quantification of the bacterial cell surface roughness based on the topographic AFM images may be done, showing again a remarkable similarity between the effects of the three different cationic antimicrobials on the cell surface (see Figure 5).

In summary, we have demonstrated for a *S. epidermidis* strain, that the antibacterial action of three different cationic antimicrobials in solution initially occurs with a similar mechanism, involving roughening of the bacterial cell surface, followed by complete disruption and detachment of the adhering organisms from a substratum surface. Based on the chemical similarity between antimicrobial peptides and QACs, it is suggested to consider QAC as synthetic antimicrobial peptides.

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